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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Clifford P. Stanners et al.
Title: CEA/NCA-BASED DIFFERENTIATION CANCER THERAPY
Attorney Docket No.: 186.009US1

PATENT APPLICATION TRANSMITTAL

BOX PATENT APPLICATION
Commissioner for Patents
Washington, D.C. 20231

We are transmitting herewith the following attached items and information (as indicated with an "X"):

X Return postcard.
X Utility Patent Application **CONTINUTATION** of Prior International Application No. PCT/CA99/00119 under 35 U.S.C. §111(a):
X Specification (27 pgs, including claims numbered 1 through 11 and a 1 page Abstract).
X Formal Drawing(s) (15 sheets).
X Unsigned Combined Declaration and Power of Attorney (4 pgs).
X Preliminary Amendment (4 pgs).

The filing fee (NOT ENCLOSED) will be calculated as follows:

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Clifford P. Stanners et al.	Examiner: Unknown
Serial No.:	Unknown	Group Art Unit: Unknown
Filed:	Herewith	Docket: 186.009US1
Title:	CEA/NCA-BASED DIFFERENTIATION CANCER THERAPY	

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION
Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to examination of the above-identified patent application, please amend as follows.

IN THE SPECIFICATION

On the first page, after the title, please insert:

--Cross-Reference to related Applications

This application is a continuation of International Patent Application No. PCT/CA99/00119, filed on February 11, 1999, which in turn is an international filing of Canadian Patent Application No. 2,224,129 filed on February 12, 1998, all of which are incorporated herein by reference.--

IN THE CLAIMS

Please amend as follows:

Please cancel claims 1-11.

12. (NEW) An Anti-CEA/NCA antibody which specifically interacts with a subdomain of CEA/NCA, wherein said subdomain is selected from the group consisting of sequences G_{30}YSWYK, N_{42}RQII, and Q_{80}ND.

13. (NEW) The antibody of claim 12, wherein said antibody releases a CEA/NCA-imposed inhibition of differentiation and/or apoptosis in CEA/NCA-producing primary and/or secondary tumor cells.

14. (NEW) A method for selecting a peptide or peptide-derived mimetics which can modulate a differentiation-blocking activity associated with a subdomain of CEA/NCA in a malignant tumor, wherein said subdomain is selected from the group consisting of sequences G_{30}YSWYK; N_{42}RQII; Q_{80}ND; sequences including epitopes of 3 to 6 amino acids in the N-terminal 107 amino acid domain; and sequences including epitopes of 3 to 6 amino acids in the internal A3B3 178 amino acid domain of CEA, wherein said peptide or peptide-derived mimetics is selected as a modulator of said differentiation-blocking activity, when a tumor cell incubated with said peptide or peptide-derived mimetics, displays a significantly modified differentiation status as compared to a tumor cell incubated in the absence thereof.

15. (NEW) Peptides and/or peptide-derived mimetics obtained by the method of claim 14, wherein said peptide and peptide-derived mimetics interacting with subdomains of CEA/NCA involved in the differentiation-blocking activity associated with malignant tumors, wherein said subdomains are selected from the group consisting of sequences G_{30}YSWYK, N_{42}RQII, and Q_{80}ND.

16. (NEW) A shankless anchor, which comprises a GPI anchor of CEA without the external domains thereof, wherein said GPI anchor interferes with downstream targets of endogenous CEA/NCA molecules to inhibit a differentiation-blocking activity thereof when administered to a primary or secondary tumor cell.

17. (NEW) A method to restore endogenous integrin function, which comprises:
an administration of a monoclonal antibody (MAB) that reverses a CEA/NCA-induced change in integrin function; or an
administration of a peptide or peptide-derived-mimetic that mimics an effect of said MAB;
thereby inhibiting a differentiation-blocking activity of endogenous CEA/NCA molecules.

18. (NEW) The method of claim 17, wherein said integrin function includes integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$.

19. (NEW) A drug screen assay to select a pharmaceutical agent which is capable of inhibiting a differentiation-blocking activity of endogenous CEA/NCA molecules in a cell, which comprises, an incubation of said cell with a candidate agent, wherein said pharmaceutical agent is selected when said differentiation-blocking activity is significantly inhibited in the presence of said candidate agent as compared to in the absence thereof.

20. (NEW) A method for enhancing efficacy of a cytoxic drug by increasing the differentiation status of tumor cells and/or by enhancing bystander effect, whereby more differentiated tumor cells cause adjacent autonomous tumor cells to behave more as non-malignant or normal cells, said method comprising an incubation of said tumor cells with an agent which interferes with one of a subdomain of CEA/NCA selected from the group consisting of sequences G_{30}YSWYK, N_{42}RQII, and Q_{80}ND, and an integrin selected from the group consisting of $\alpha_5\beta_1$ and $\alpha_v\beta_3$, thereby increasing said differentiation status and enhancing said efficacy of said drug.

21. (NEW) The method of claim 14, wherein said subdomain is selected from the group consisting of sequences G_{30}YSWYK, N_{42}RQII, and Q_{80}ND.

22. (NEW) The method of claim 19, wherein said cell is a rat L6 myoblast expressing CEA/NCA.

23. (NEW) The method of claim 19, wherein said cell is a human Caco-2 colonocyte which aberrantly expresses a high level of CEA/NCA, and wherein said inhibition of differentiation-blocking activity can be positively correlated with a restoring of normal cellular and tissue architecture of said Caco-2 cells, upon incubation with said pharmaceutical agent.

24. (NEW) The method of claim 20, wherein said agent is selected from the group consisting of:

a) anti-CEA/NCA antibodies which specifically interact with a subdomain of CEA/NCA selected from sequences G_{30}YSWYK, N_{42}RQII, and Q_{80}ND;

b) a peptide having a sequence selected from G_{30}YSWYK, N_{42}RQII, and Q_{80}ND;

c) a peptide mimetic of b);

d) an antisense of CEA/NCA; and

e) a shankless anchor of CEA/NCA comprising a GPI anchor of CEA without the external domains thereof.

25. (NEW) A method of relieving a CEA/NCA-imposed inhibition of differentiation and/or apoptosis comprising an incubation of primary or secondary tumor cells with an agent which disrupts one of an interaction between CEA/NCA subdomains having sequences selected from G_{30}YSWYK, N_{42}RQII, and Q_{80}ND, and a functional interaction between said subdomains and integrin $\alpha_5\beta_1$ and $\alpha_v\beta_3$.

REMARKS

Claims 1 through 11 have been canceled and claims 12-25 have been newly added. Claims 12-25 are currently pending.

Respectfully submitted,

CLIFFORD P. STANNERS ET AL.

By their Representatives,

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Date August 11, 2000 By Ann M. McCrackin

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CEA/NCA-BASED DIFFERENTIATION CANCER THERAPY

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a novel cancer therapy based on the direct or indirect downregulation of endogenous CEA/NCA which plays an instrumental role in malignant progression through its differentiation-blocking activity on normal cells.

(b) Description of Prior Art

The long-term cure rates for cancers at many sites treated by present means, such as surgery, radiation and chemotherapy, are often unacceptably low. Due to the common presence of metastases derived from the primary tumor, it is impossible to treat most cancers effectively with surgery and radiation alone. Systemic chemotherapy is effective in some cases but is often too toxic to permit the use of the doses required for cure. Novel treatments based on molecular differences between cancer and normal cells are required. Such treatments would likely be non-toxic and, since they would be based on different principles from the commonly used treatments, would be expected to be synergistic with them, giving more effective combined treatment.

Tumor cells at many sites, including colon, breast, lung, cervix, ovary, stomach, bladder, pancreas and esophagus express large amounts of carcinoembryonic antigen (CEA) and/or the closely related family member, NCA, on their surfaces. The expression of these glycoproteins, especially CEA, in normal cells is very limited. This represents the basis for the wide clinical use of CEA as a blood tumor marker. Since the majority of human cancers show up-regulation of CEA/NCA, any therapy based on this fact has potential application to an immense number of cancer patients.

This upregulation in so many types of cancer led us to suggest that CEA and NCA could actually contribute directly to tumorigenicity. We first showed that CEA (Benchimol et al, Cell 57:327-334, 1989) and NCA (Zhou et al, Cell Growth Differ. 1:209-215, 1990) function as intercellular adhesion molecules. Although CEA had been previously considered as an inert marker of tumorigenicity, we suggested that inappropriate CEA/NCA expression in cells still capable of proliferation could cause a distortion of tissue architecture (which is determined by adhesion molecules) and an inhibition of terminal differentiation that normally removes cells from the pool of cells with potential to proliferate, thus contributing directly to malignant progression.

In agreement with this hypothesis, we have shown that CEA and NCA expression in transfected myoblasts can inhibit terminal myogenic differentiation and promote tumorigenicity. Peptides representing the adhesion domains of CEA can release the myogenic differentiation block in CEA-transfected myoblasts, indicating the necessity of CEA-CEA interactions for the inhibition of terminal differentiation.

In the present invention, the inhibition of terminal differentiation by CEA/NCA over-expression has been demonstrated to apply to the adipogenic differentiation of mouse fibroblasts, to the neuronal differentiation of mouse embryonal carcinoma cells and to the differentiation and polarization of human colonocytes. CEA/NCA over-expression has also been shown to distort tissue architecture and to inhibit anoikis (apoptosis of anchorage-free cells). The inhibition of differentiation depends on both interactions between the external domains of CEA and on the presence of a CEA-determined glycophosphatidyl-inositol (GPI) membrane anchor. Novel treatments based

on reversal of the carcinogenetic effects of CEA/NCA would be highly desirable since they would be precisely targeted to tumor cells expressing these molecules and should therefore be applicable to a large proportion of human cancers. Reversal can be achieved by interference with certain subdomains of CEA and NCA that are required for the differentiation-blocking activity, by downregulating the cellular production of these molecules and by three other means, as delineated below.

It would be highly desirable to provide a novel cancer therapy based on the direct or indirect downregulation of endogenous CEA/NCA which plays an instrumental role in malignant progression, through its differentiation-blocking activity on normal cells.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a novel cancer therapy based on interference with the differentiation-blocking activity of CEA/NCA or on downregulating the production of CEA/NCA, which we show plays an important role in malignant progression. This treatment based on this property of CEA/NCA is precisely targeted to tumor cells expressing these molecules and is therefore non-toxic and applicable to a large proportion of human cancers.

In accordance with the present invention, there exists five (5) different routes for interference with the tumorigenic effects of CEA/NCA:

1. Antibodies raised against subdomains of CEA/NCA, small peptides and derived mimetics (organic structures that mimic peptides) which all interact with precise subdomains of CEA/NCA that are involved in the differentiation-blocking activity associated with malignant progression. The antibodies, peptides and

derived mimetics when administered to cancer patients will restore differentiation and thus inhibit tumorigenicity.

2. Antisense cDNA constructs, ribozymes and oligonucleotides reduce the expression of CEA/NCA when administered directly or by gene therapy to a cancer patient, which restores differentiation and thus inhibits tumorigenicity.

3. Cell surface receptors involved in extracellular matrix (ECM) binding, integrins, are involved in the differentiation-blocking activity of CEA/NCA molecules. The particular integrins, including $\alpha_5\beta_1$, and $\alpha_v\beta_3$ have been identified. Inhibition of the changes that these molecules undergo as a result of CEA/NCA activity could release the CEA/NCA-imposed differentiation block. Certain monoclonal antibodies directed against these particular integrins have this effect and when administered to patients will restore differentiation and thus inhibit tumorigenicity.

4. "Shankless anchors", consisting of the GPI anchor of CEA without the external domains, interfere with the differentiation-inhibiting activity of CEA/NCA and can be administered directly to a cancer patient. The "shankless anchors" inhibit the differentiation-blocking activity of the endogenous CEA/NCA molecules by competing with CEA/NCA for the elements of the molecular pathway required for the CEA/NCA effect.

5. Agents that interfere with the signaling process between CEA/NCA molecules activated by

self association and the aforementioned integrins, thus interfering with the changes in the latter that inhibit differentiation, could release the CEA/NCA-imposed differentiation block.

All these routes lead to new useful clinical agents capable of removing tumor cells by inducing their terminal differentiation, thus effectively killing tumor cells by a mechanism distinct from that of cytotoxic drugs.

In accordance with the present invention there is provided an inhibiting CEA/NCA sequence, which comprises antisense mRNA sequences which hybridize to at least one domain of CEA/NCA selected from the group consisting of the cDNA sequences of CEA and NCA to reduce the expression of endogenous CEA/NCA when administered to a cancer patient.

In accordance with the present invention there is also provided inhibiting CEA/NCA nucleotide sequences, wherein the sequence is an antisense cDNA, an antisense oligonucleotide or an antisense ribozyme containing CEA/NCA antisense nucleotide sequences.

In accordance with the present invention there is also provided anti-CEA/NCA antibodies, which comprise antibodies raised against subdomains of CEA/NCA involved in the differentiation-blocking activity associated with tumorigenicity, wherein the subdomains are selected from the group consisting of the sequences G_{30}YSWYK, N_{42}RQII, Q_{80}ND and other sequences in the N terminal 107 amino acid domain, and sequences in the internal A3B3 domain of CEA.

In accordance with the present invention there is also provided peptides and peptide-derived mimetics, which comprise peptide and peptide-derived mimetics interacting with subdomains of CEA/NCA involved in the

differentiation-blocking activity associated with tumorigenicity, wherein the subdomains are selected from the group consisting of the sequences G_{30}YSWYK, N_{42}RQII, Q_{80}ND and other sequences in the N-terminal 107 amino acid domain, and sequences in the internal A3B3 domain of CEA.

The present invention includes also combinations of peptides representing these subdomains in which the peptides are free or linked together with polyethylene glycol molecules.

In accordance with the present invention there is also provided a shankless anchor, which comprises a GPI anchor of CEA without the external peptide domains attached, wherein the GPI anchor interferes with downstream targets of endogenous CEA/NCA molecules to inhibit the differentiation-blocking activity of the endogenous CEA/NCA molecules.

In accordance with the present invention there is also provided a method to restore endogenous integrin function including integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, which comprises the steps of:

a) administration of monoclonal antibodies that reverse the CEA/NCA-induced changes in integrin function; and

b) administration of peptides/mimetics that mimic the effect of the mAbs;

thereby inhibiting the differentiation-blocking activity of the endogenous CEA/NCA molecules.

In accordance with the present invention there is also provided a drug screen assay utilizing CEA/NCA-expressing transfectants of rat L6 myoblasts to determine pharmaceutical agents which are capable of inhibiting the signaling process required for differentiation-blocking activity of the endogenous CEA/NCA molecules, which comprises the steps of:

a) screening for agents capable of releasing the myogenic differentiation block in rat L6 cells expressing CEA/NCA; and

b) screening for agents capable of restoring normal cellular and tissue architecture to human Caco-2 colonocytes aberrantly expressing high levels of CEA/NCA.

In accordance with the present invention there is also provided the use of the anti-CEA/NCA antibodies, the peptides and peptide-derived mimetics, the inhibiting CEA/NCA sequence, or the shankless anchor of the present invention, to enhance efficacy of other anti-cancer treatments by increasing the differentiation status of a tumor and by enhancing the bystander effect; whereby more differentiated tumor cells cause more adjacent autonomous tumor cells to behave more as non-malignant or normal cells. Other treatment modes will not be required to kill as many tumor cells in order to be efficacious.

In accordance with the present invention there is also provided anti-CEA/NCA antibodies, the peptides and peptide-derived mimetics, the inhibiting CEA/NCA sequence, or the shankless anchor of the present invention, to restore anoikis/apoptosis to levels of non-malignat or normal cells, thereby increasing the efficacy of all other cytotoxic chemotherapeutic drugs which depend on apoptosis for killing cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the effect of CEA/NCA overexpression in normal epithelial cells of colonic crypts which contributes to the development of a malignant tumor. The different sites of action of the routes 1, 2, 3, 4 and 5 of the present invention as described above are indicated;

Fig. 2 illustrates that overexpression of NCA in human colorectal carcinoma cells, SW-1222, blocks the formation of glandular-like structures of polarized cells with central lumens in monolayer culture;

Fig. 3 (top) illustrates that everexpression of NCA blocks the formation in collagen gels of glandular spheroids consisting of radially arranged polarized colonocytes with central lumens;

Fig. 3 (bottom) illustrates that overexpression of CEA and NCA in human colorectal carcinoma cells, Caco-2, blocks their polarization in monolayer culture leading to tumor-like multilayered structures with circumferential expression of CEA;

Fig. 4 illustrates that dome formation, due to vectorial transport of solvent from apical to basolateral surfaces of colonocytes and an indicator of polarization, is strongly inhibited by over-expression of CEA/NCA in Caco-2 colonocytes;

Fig. 5 illustrates that overexpression of NCA in SW-1222 cells causes loss of colonic glandular crypt formation with polarized cells facing a central lumen in a tissue architecture assay *in vivo*;

Fig. 6 illustrates that NCA overexpression inhibits anoikis (apoptosis) of SW-1222 cells cultured in suspension;

Fig. 7 illustrates that down-regulation of CEA in SW-1222 cells results in more normal, less tumorigenic cellular characteristics;

Fig. 8 illustrates the quantitative evidence that down-regulation of CEA in SW-1222 cells results in more glandular spheroids with recognizable central lumens;

Fig. 9 illustrates the subdomains in the NH2-terminal N domain of CEA that are required for intercellular adhesion and for the myogenic

differentiation block. The quantitative effects of mutations in these subdomains on CEA-mediated adhesion and on CEA-mediated myogenic differentiation block are also shown;

Fig. 10 illustrates photomicrographs of L6 transfectants indicated in Fig. 9, showing the extent of myogenic differentiation in some of the subdomain mutants;

Fig. 11 illustrates the effects of the addition of small cyclic peptides, that include the amino acid sequences indicated in the three N domain subdomains, on the myogenic differentiation of rat L6 myoblasts. Myogenic differentiation is indicated by positive labelling with fluorescent anti-myosin antibody. CEA production blocks myogenic differentiation completely whereas all three peptides can release the CEA-imposed differentiation block;

Fig. 12 illustrates that substitution of the normal trans-membrane domain of the BGPa molecule (Tm) with the GPI membrane domain of CEA converts BGPa from a molecule that has no effect on myogenic differentiation (upper micrograph) to one (denoted BC-2) that inhibits myogenic differentiation completely (lower micrograph); and

Fig. 13 illustrates that substitution of the carboxy-terminal GPI-determining domain of NCAM-125 with the GPI domain of CEA giving the hybrid construct, NCAM 125-CEA, converts NCAM from a molecule that has no effect on myogenic differentiation to one that inhibits myogenic differentiation completely.

DETAILED DESCRIPTION OF THE INVENTION

Although CEA had been previously considered as an inert marker of tumorigenicity, we suggested (Benchimol et al, 1989) that inappropriate CEA/NCA expression in cells still capable of proliferation

could cause a distortion of tissue architecture and an inhibition of terminal differentiation which normally removes cells from the pool of cells with potential to proliferate, thus contributing directly to malignant progression.

Fig. 1 illustrates the effect of CEA/NCA in normal cells which contributes to the development of a malignant tumor and the different sites of action of the routes 1, 2, 3, 4 and 5 of the present invention as described above.

The above hypothesis was tested in several model systems: CEA expression in rat myoblasts (by transfection with CEA cDNA) was shown to block terminal myogenic differentiation completely (Eidelman et al, J. Cell Biol. 123:467-475, 1993); NCA had the same effect whereas a CEA family member that is down-regulated in cancer (BGP) had no effect (Rojas et al, Cell Growth Differ. 7:655-662, 1996); CEA expression inhibits terminal adipogenic differentiation of mouse adipocytes; CEA and NCA, but not BGP, expression inhibits the neuronal differentiation of mouse embryonal carcinoma cells; finally, human colorectal carcinoma cell lines, SW-1222 and Caco-2, that retain differentiation and polarization capacity, when forced by transfection to over-express CEA and NCA, lose their ability to form colonic crypt-like glandular structures with central lumens in both monolayer and spheroid culture and lose their ability to polarize, closely resembling the more progressed human colorectal carcinomas removed from patients (Figs. 2, 3 and 4). Human colorectal carcinoma cell line SW-1222, stably transfected with empty expression vector [SW(Hygro)] or with vector containing NCA cDNA and thereby overexpressing NCA by 5-10 fold (in proliferating cells) [SW-NCA↑], grown in monolayer culture show

lumens with radially arranged polarized cells in the case of the control SW(Hygro) cells but not in the case of the SW-NCA↑ cells (top, Fig. 2). Polarization of the control SW(hygro) cells is shown by the presence of rings of villin staining localized with microvilli at the lumens (bottom, Fig. 2). Thus the residual degree of glandular differentiation exhibited by the human SW-1222 colonocytes in monolayer is completely inhibited by over-expression of NCA (Fig. 2, inset).

In Fig. 3(top), SW-1222(Hygro) cells grown in suspension in collagen gels form glandular spheroids consisting of radially arranged polarized colonocytes with central lumens selectively stained with anti-NCA mAb, whereas SW-NCA↑ cells form only irregular non-polarized cell masses with generalized NCA staining and no lumens. In Fig. 3(bottom), human colorectal carcinoma cell line Caco-2, stably transfected with empty expression vector [Caco(Hygro)] or with vectors containing CEA cDNA and NCA cDNA [Caco CEA/NCA↑] and thereby overexpressing CEA & NCA by 20 fold (in proliferating cells), were cultured for 17 days on solid support. Cultures were sectioned vertically and stained with hematoxylin to show cell nuclei or with anti-CEA mAb to show the polarized expression of CEA at the apical upper surfaces of the monolayer of columnar colonocytes. Note the multilayered (stratified) configuration with circumferential CEA staining of the Caco CEA/NCA↑ cells. Thus CEA/NCA over-expression destroys the normal monolayered architecture of polarized columnar colonocytes of Caco-2, giving a tissue architecture closely mimicking that of colon carcinomas.

In Fig. 4B, dome formation, due to vectorial transport of solvent from apical to basolateral surfaces of colonocytes and an indicator of

polarization, is strongly inhibited by over-expression of CEA/NCA in Caco-2 colonocytes. High levels of CEA expressed in Caco-2 cells transfected with CEA cDNA alone, Caco-CEA↑P3, also prevented the formation of domes. These cells, after culture in the absence of the selective agent (Hygromycin) resulting in loss of all CEA over-expression (see inset), renamed Caco-CEA - Hygro, dramatically regain their ability to form domes (Fig. 4C). Domes can be seen as raised circular areas of cells in the photomicrograph of control Caco(Hygro) cells (Fig. 4A, upper left) but not in Caco CEA/NCA↑ cells (Fig. 4A, upper right).

In an assay, developed in our laboratory, that tests the ability of human colonocytes to conform to normal tissue architecture (Ilantzis & Stanners, In Vitro Cell. Dev. Biol.- Animal 33: 50-61, 1997), over-expression of NCA causes a marked deterioration of their capacity to form colonic crypts (Fig. 5). Sections of minicolons obtained from growth of mixed aggregates of normal fetal rat colonocytes and 1% SW(Hygro) or SW-NCA↑ cells for 7-10 days under the kidney capsule of the nude mouse are shown in Fig. 5. The SW-1222 cells were identified by staining with an anti-CEA mAb. Note the dramatic deterioration of the quasi-normal tissue architecture of the SW(Hygro) cells as exemplified by rings of staining that are absent in the SW-NCA↑ cells over-expressing NCA.

In fact, we have shown that the cell surface levels of CEA and NCA on purified colonocytes from freshly excised colon carcinomas and adjacent normal tissue from patients, measured by FACS analysis, are markedly higher on the tumor cells relative to the normal cells and inversely correlated with the degree of differentiation of the tumors (Ilantzis et al, Lab Invest. 76(5):703-716, 1997); this represents clinical

evidence in support of our hypothesis. Even microadenomas, the early precursors of colonic carcinomas showed upregulation of CEA and NCA in direct relation to their degree of dysplasia (Ilantzis et al, 1997).

In agreement with the suggestion that these effects of CEA/NCA over-expression could drive malignant progression, both the rat myoblasts (Screaton et al, J. Cell Biol. 137:939-952, 1997) and human CaCo-2 colonocytes transfected with CEA/NCA, but not with the empty transfection vector, produced tumors in nude mice with a strikingly reduced latent period.

The above results support the contention that CEA and NCA are general inhibitors of terminal cellular differentiation. We have evidence that they achieve this by interference with the function of integrins responsible for cell/extracellular matrix interactions. The latter interactions are known to be required for many different types of cellular differentiation. Furthermore, we have direct evidence that these CEA/NCA-induced perturbations in integrin function inhibit anoikis, the apoptotic process that is employed to destroy cells that do not conform to normal tissue architecture (Fig. 6). CEA/NCA over-expression inhibits anoikis. SW-1222 cells attached to a solid support, stained with DAPI to show nuclear morhology, demonstrate whole nuclei (upper left). When incubated in suspension, parental SW-1222 cells, SW(hygro) cells and SW-CEA↓ cells (with CEA expression down-regulated) all show fragmented nuclei, indicative of anoikis (apoptosis). SW-NCA↑ cells, expressing much higher levels of NCA, show whole nuclei when incubated in suspension, thus not demonstrating anoikis. The overproduction of CEA/NCA affects the function of two particular integrins, $\alpha_5\beta_1$ and $\alpha_v\beta_3$; monoclonal

antibodies directed against these integrins will reverse the inhibition of anoikis mediated by CEA/NCA.

The inhibition of apoptosis by the over-production of CEA/NCA is an alteration that will contribute to the development of cancer. It will also lead to resistance to cell killing by cytotoxic agents used in chemotherapy, which our results with certain such agents indicates. Thus, inhibition of these effects of CEA/NCA will lead to increased sensitivity to cytotoxic chemotherapeutic drugs.

The overall picture, then, is one of CEA/NCA-induced inhibition of terminal cellular differentiation, cellular polarization and anoikis, and an accompanying loss of tissue architecture. The net effect of these CEA/NCA-induced cellular changes is to promote malignancy and to produce resistance to cell killing chemotherapeutic agents.

1. Antibodies, Peptides and Mimetics:

The myogenic differentiation-blocking activity of CEA, at least, can be reversed by interference with the adhesion domains of CEA (the N and A3B3 domains - Zhou et al, J. Cell Biol. 122: 951-960, 1993) using domain-specific peptides made in bacteria or by a deletion in the N-terminal domain (Eidelman et al, J. Cell Biol. 123:467-475, 1993), indicating the necessity of CEA-CEA interaction for the effect. In fact, the ΔNCEA deletion mutant that is defective in its ability to effect a myogenic differentiation block can be potentiated by the application of cross-linking monoclonal antibodies. We therefore presume that it is CEA-CEA binding leading to clustering on the cell surface that is required for the differentiation block. Further work has shown that the glycophosphatidyl

inositol (GPI) membrane anchor of CEA is also required for the myogenic differentiation block.

The precise subdomains in the N domain of the CEA molecule responsible for intercellular adhesion and for the myogenic differentiation block (and, by implication, other types of differentiation block) have been identified. These are NRQII, starting at amino acid #42 in the N domain of CEA (where the numbering begins at the first amino acid of the mature protein) which, when deleted or mutated to NRRIV (Q44R&I46V) or DRQII (N42D), abrogate both intercellular adhesion in transfected CHO-derived cells and the myogenic differentiation block; in addition, mutations giving amino acid substitutions at QND, starting at amino acid #80 in the N domain, giving QAD (N81A) or QNN (D82N) completely remove the ability of CEA to block myogenic differentiation without affecting its ability to mediate intercellular adhesion in CHO-derived cells (Figs. 9 and 10). Mutations in a third subdomain, GYSWYK, starting at amino acid #30 in the N domain of CEA, also can remove the ability of CEA to block myogenic differentiation. The subdomains in the NH_2-terminal N domain of CEA that are required for intercellular adhesion of stable transfectants of CHO-derived LR cells and for the myogenic differentiation block of stable transfectants of rat L6 myoblasts are shown in Fig. 9. The positions of the 3 subdomains in the N domain of CEA that are required for adhesion and differentiation block are shown (top, Fig. 9). The effects of mutations in these subdomains on CEA-mediated adhesion, indicated by the % of cells remaining as single cells after incubation in suspension for 2 hrs, and on the CEA-mediated myogenic differentiation block, indicated by the % of nuclei in fused cells, are shown (bottom, Fig. 9).

Photomicrographs of L6 transfectants indicated in Fig. 9, showing the extent of myogenic differentiation in some of the subdomain mutants after growth under differentiation conditions are presented in Fig. 10. The control L6 cells transfected with vector alone, L6-Neo, show extensive differentiation, whereas L6 cells transfected with CEA cDNA (L6-CEA) show none. Deletion of NRQII and especially point muations at D82 and Q44+I46 show release of the CEA-imposed differentiation block.

Thus the adhesive and differentiation-blocking activities of CEA can be separated, allowing the possibility of precise interference with the differentiation-blocking activity.

In fact, cyclic peptides including the sequences GYSWYK, NRQII and QND of the three subdomains of the CEA N domain have been applied to L6 myoblasts producing CEA and have dramatically released the CEA-imposed block in myogenic differentiation (Fig. 11). A similar release of the CEA-imposed myogenic differentiation block has been obtained by addition of monoclonal antibodies A20.12.2 (Zhou et al., Cancer Res. 53: 3817-3822, 1993) and others that we have shown to bind to an epitope including the YK residues of GYSWYK and the N residue of NRQII.

We thus propose to use peptides or mimetics representing these subdomains or monoclonal antibodies that bind to them that block the necessary CEA-CEA or NCA-NCA intermolecular interactions for the tumorigenic effects of CEA/NCA as agents for clinical use.

2. Antisense Agents:

Importantly, human SW-1222 colon carcinoma cells transfected with a defective mutant of CEA and thereby actually producing less than normal levels of

CEA, denoted SW-CEA↓, were more differentiated than the parental cells, in that they exhibited a more normal flat morphology in monolayer culture (Fig. 7) and more readily formed glandular spheroids in collagen gels (Fig. 8), thus indicating the possibility of reversal of the tumorigenicity of colonic carcinoma cells by forcing the down-regulation of CEA/NCA. Micrographs of control SW-1222 and SW-CEA↓ monolayer cultures are presented in Fig. 7, showing that down-regulation of CEA (see FACS profiles for cells stained with fluorescent CEA-specific mAb, D-14) results in a more normal, flatter morphology. The SW-CEA↓ line was obtained by stable transfection with the defective N-domain deletion mutant, ΔNCEA (Eidelman et al, 1993) (Fig. 7).

Such down regulation could be achieved by the application of CEA/NCA anti-sense oligonucleotides or anti-sense ribozymes to tumors or gene therapy with CEA/NCA antisense cDNA constructs.

3. Regulation of integrin function

We have recently shown that CEA/NCA over-expression inhibits terminal differentiation in so many different types of cells by perturbing a molecular process common to all, that of interaction with the extra-cellular matrix (ECM). The major class of cellular receptors responsible for ECM interactions are the integrins; integrin-ECM interactions are known to be involved in many types of differentiation, in the maintenance of tissue architecture and in anoikis (apoptosis). The particular integrin disturbed in its function by CEA/NCA expression (but not by BGP or other controls) is $\alpha_5\beta_1$, as shown by reversal of the inhibitory effect of CEA/NCA on anoikis of suspended rat L6 myoblasts and human Caco-2 cells by a monoclonal

antibody against this integrin. This mAb or peptides/mimetics that mimic its effects on $\alpha_5\beta_1$ function could be administered to patients bearing CEA/NCA expressing tumors and would be expected to release the differentiation block imposed by CEA/NCA. In mouse P19 embryonal carcinoma cells, the integrin affected is $\alpha_v\beta_3$. The agents described above in routes 1, 2, 3, 4 and 5 of the present invention should cause CEA/NCA over-expressing tumor cells to differentiate terminally, thus removing them as potential colonizing cells in the body.

4. Shankless Anchors:

We have shown that the structural features of the CEA molecule required for the differentiation are as follows: first, external domains capable of self association and, second, attachment of these to the hydrophobic carboxy-terminal domain of CEA; the latter domain is normally cleaved during processing events resulting in the formation of a GPI membrane anchor. Thus the trans-membrane linked BGPa member of the CEA family, normally without effect on myogenic differentiation, can be converted to one that blocks differentiation by the addition of the CEA GPI domain (Fig. 12). Substitution of the normal trans-membrane domain of the BGPa molecule (Tm), including its cytoplasmic domain, with the GPI membrane domain of CEA (GPI) converts BGPa from a molecule that has no effect on myogenic differentiation (upper micrograph) to one (denoted BC-2) that inhibits myogenic differentiation completely (lower micrograph). FACS profiles showing cell surface levels of BGPa and BC-2 indicate equivalent levels of expression (Fig. 12). Conversely, the substitution of the GPI anchor of CEA with the transmembrane anchor of BGPa, converts CEA from a

molecule that blocks myogenic differentiation into one that has no effect. Strikingly, the GPI membrane-linked NCAM splice isoform, NCAM-125, which has no effect on myogenic differentiation, can also be converted to a molecule with differentiation blocking capacity by the addition of the CEA GPI domain (Fig. 13). The GPI-linked NCAM (neural cell adhesion molecule) splice isoform with the muscle-specific domain (MSD), NCAM 125, even at relatively high levels of cell surface expression in rat L6 stable transfectants, has no effect on myogenic differentiation. Substitution of its own carboxy-terminal GPI-determining domain (open circle) with the GPI domain of CEA (full circle) giving the hybrid construct, NCAM 125-CEA, however, converts it into a molecule that inhibits myogenic differentiation completely (Fig. 13).

Strategy 1. depends on interference with the binding domains of CEA which are the self-binding domains naturally associated with the CEA GPI anchor. The present strategy is targeted to the GPI domain itself. CEA molecules lacking binding domains, consisting of the GPI anchor alone, with little or no attached peptide ("shankless anchors"), can be generated by enzymatic cleavage or by the use of CEA cDNA constructs with deleted binding domains. These can be applied directly to cells blocked in differentiation by CEA/NCA and, as has been shown for other GPI-linked molecules, should successfully embed themselves from the external milieu into the membrane of the cells. Inhibition with the differentiation-blocking activity of the endogenous CEA/NCA molecules is anticipated via competition by the CEA shankless anchors for elements of the molecular pathway required for the CEA/NCA effect. Release of the CEA-mediated myogenic differentiation block has, in fact, been observed by

co-transfection with CEA cDNA constructs producing CEA molecules with intact GPI anchors but defective external binding domains.

The above inhibitory effect of GPI anchors could be applied in principle to inhibit the activity of any GPI-linked molecule. This extends the potential utility of the present invention to include many types of GPI-linked molecules with a wide range of biomedical effects.

Development of the present invention over the next year

1. Antisense oligonucleotides, ribozymes and cDNA constructs will be prepared and tested for their ability to reduce the expression of CEA/NCA in SW-1222 (NCA↑) cells and Caco-2 (CEA/NCA↑) cells. The effects on the cellular and tissue architecture and tumorigenicity of these transfectants so treated will be measured, expecting a reversal to more normal behaviour.
2. Peptide mimetics against the differentiation-blocking subdomains of CEA will be developed and tested for their ability to release the myogenic differentiation block and to reduce the tumorigenicity of L6 myoblast transfectants expressing CEA. The mimetics will also be tested for ability to restore normal cell and tissue architecture and reduce tumorigenicity of SW-1222 (NCA↑) and Caco-2 (CEA/NCA↑) cells.
3. CEA shankless anchors will be prepared and applied to L6 myoblast transfectants expressing CEA, testing for their ability to release the CEA-imposed differentiation block.
4. The mAb against $\alpha_5\beta_1$ will be tested for its ability to reverse all of the effects of CEA

expression on the L6 myoblasts, including impairment of binding to ECM and the differentiation block. The test will be extended to SW-1222 (NCA↑) and Caco-2 (CEA/NCA↑), looking for restoration of normal cell and tissue architecture.

The present invention will be more readily understood by referring to the following example which is given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Peptide/mimetic, monoclonal antibody or drug screening assay

L6 rat myoblasts transfected with CEA/NCA cDNA and thereby blocked in their differentiation provide the most sensitive assay for screening agents capable of releasing the differentiation block.

1. L6 (CEA/NCA) cells producing CEA or NCA are seeded into tissue culture plates containing multiple wells and cultured until forming a confluent monolayer.
2. The medium is changed to a medium poor in growth factors (DMEM plus 2% horse serum) that stimulates differentiation. At the same time the agent to be tested is added at a series of concentrations to an appropriate number of cultures in the wells.
3. The culture plate is incubated for 5-7 days. If the agent being tested is unstable, additional agent is added during this incubation period.
4. At the end of the incubation period, the medium is removed and the cultures stained with hematoxylin. Release of the CEA/NCA-imposed differentiation block is easily assessed by the

presence of mutinucleated giant cells that also stain positively with anti-myosin antibody (see Figs. 10 and 11) and quantitated by the percentage of total nuclei in cells with >3 nuclei.

5. Agents that give high levels of myogenic differentiation are then tested for effects on human colonocytes aberrantly expressing high levels of CEA/NCA. The simplest assays are the test for formation of glandular structures of polarized cells by SW-1222 (NCA↑) cells in monolayer (see Fig. 2) and the assay for dome formation and polarization of Caco-2 (CEA-NCA↑) cells (see Fig. 4).

6. Agents that reverse the CEA/NCA-mediated inhibition of cellular polarization, tissue architecture and differentiation of human colorectal carcinoma cell lines are then tested for their ability to inhibit the tumorigenicity, the formation of metastases and reverse the undifferentiated characteristics of the same cell lines injected into the cecum or spleen of nude mice. They are also tested for their efficacy in restoring normal tissue architecture in our mouse tissue architecture assay (Ilantzis & Stanners, In Vitro Cell. Dev. Biol.- Animal 33: 50-61, 1997). Finally, successful agents are administered to patients bearing cancers that over-produce CEA/NCA, with the expectation that they will block the growth of tumors and their derived metastases by forcing them to differentiate.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications

and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. Anti-CEA/NCA antibodies, which comprise antibodies raised against subdomains of CEA/NCA involved in differentiation-blocking activity associated with tumorigenicity, wherein said subdomains are selected from the group consisting of sequences G_{30}YSWYK (SEQ ID NO:1), N_{42}RQII (SEQ ID NO:2), Q_{80}ND and other sequences in the N terminal 107 amino acid domain, and sequences in the internal A3B3 178 amino acid domain of CEA.

2. The antibodies of claim 1, wherein said antibodies release CEA/NCA-imposed differentiation block in CEA/NCA-producing tumors and their metastases in a cancer patient.

3. Peptides and peptide-derived mimetics, which comprises peptide and peptide-derived mimetics interacting with subdomains of CEA/NCA involved in the differentiation-blocking activity associated with malignant tumors, wherein said subdomains are selected from the group consisting of sequences G_{30}YSWYK (SEQ ID NO:1), N_{42}RQII (SEQ ID NO:2), Q_{80}ND and other sequences in the N-terminal 107 amino acid domain, and sequences in the internal A3B3 178 amino acid domain of CEA.

4. An inhibiting CEA/NCA sequence, which comprises antisense cDNA, oligonucleotide or ribozyme sequences which hybridize to at least one domain of CEA/NCA selected from the group consisting of mRNA sequences of CEA and NCA which reduces expression of CEA/NCA in tumors and metastases when administered to a cancer patient.

5. The inhibiting CEA/NCA sequence of claim 1, wherein said sequence is an antisense cDNA, an antisense oligonucleotide or an antisense ribozyme.

6. A shankless anchor, which comprises a GPI anchor of CEA without the external domains, wherein said GPI anchor interferes with downstream targets of endogenous CEA/NCA molecules to inhibit differentiation-blocking activity of endogenous CEA/NCA molecules when administered to a cancer patient.

7. A method to restore endogenous integrin function, which comprises the steps of:

a) administration of monoclonal antibodies that reverse EA/NCA-induced changes in integrin function; and

b) administration of peptides/mimetics that mimics the effect of the mAbs;

thereby inhibiting differentiation-blocking activity of the endogenous CEA/NCA molecules.

8. The method of claim 7, wherein said integrin function includes integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$.

9. A drug screen assay utilizing CEA/NCA-expressing transfectants of rat L6 myoblasts to determine pharmaceutical agents which are capable of inhibiting signaling process required for differentiation-blocking activity of the endogenous CEA/NCA molecules, which comprises the steps of:

a) screening for agents capable of releasing myogenic differentiation block in rat L6 cells expressing CEA/NCA; and

b) screening for agents capable of restoring normal cellular and tissue architecture to human Caco-2

colonocytes aberrantly expressing high levels of CEA/NCA.

10. The use of the anti-CEA/NCA antibodies of claims 1 and 2, the peptides and peptide-derived mimetics of claim 3, the inhibiting CEA/NCA sequence of claims 4 and 5 or the shankless anchor of claim 6, to enhance efficacy of other anti-cancer treatment by increasing differentiation status of a tumor and by enhancing bystander effect; whereby more differentiated tumor cells cause more adjacent autonomous tumor cells to behave more as non-malignant or normal cells.

11. The use of the anti-CEA/NCA antibodies of claims 1 and 2, the peptides and peptide-derived mimetics of claim 3, the inhibiting CEA/NCA sequence of claims 4 and 5 or the shankless anchor of claim 6, to restore anoikis/apoptosis to levels of non-malignant or normal cells, thereby increasing efficacy of all other cytotoxic chemotherapeutic drugs which depend on apoptosis for killing cells.

ABSTRACT OF THE INVENTION

The present invention relates to a novel cancer therapy based on interference with the function or on downregulation of overproduced CEA/NCA, which plays an instrumental role in tumorigenesis and malignant progression through its differentiation-blocking activity. More precisely, there is provided three short amino acid sequence subdomains in the N domain of CEA and NCA that, when applied as peptides, peptide mimetics or anti-sudomain monoclonal antibodies to malignant tumors overproducing CEA/NCA, induce them to differentiate, thereby inhibiting their ability to grow and increasing the efficacy of other modes of treatment. Four other means of releasing the CEA/NCA-imposed differentiation block are also provided. The enhanced differentiation status of cancers induced by these CEA/NCA-based novel modes of treatment is expected to increase the efficacy of virtually any other mode of treatment by enhancing the bystander effect, whereby more differentiated cancer cells normalize the behaviour of adjacent less differentiated cancer cells.

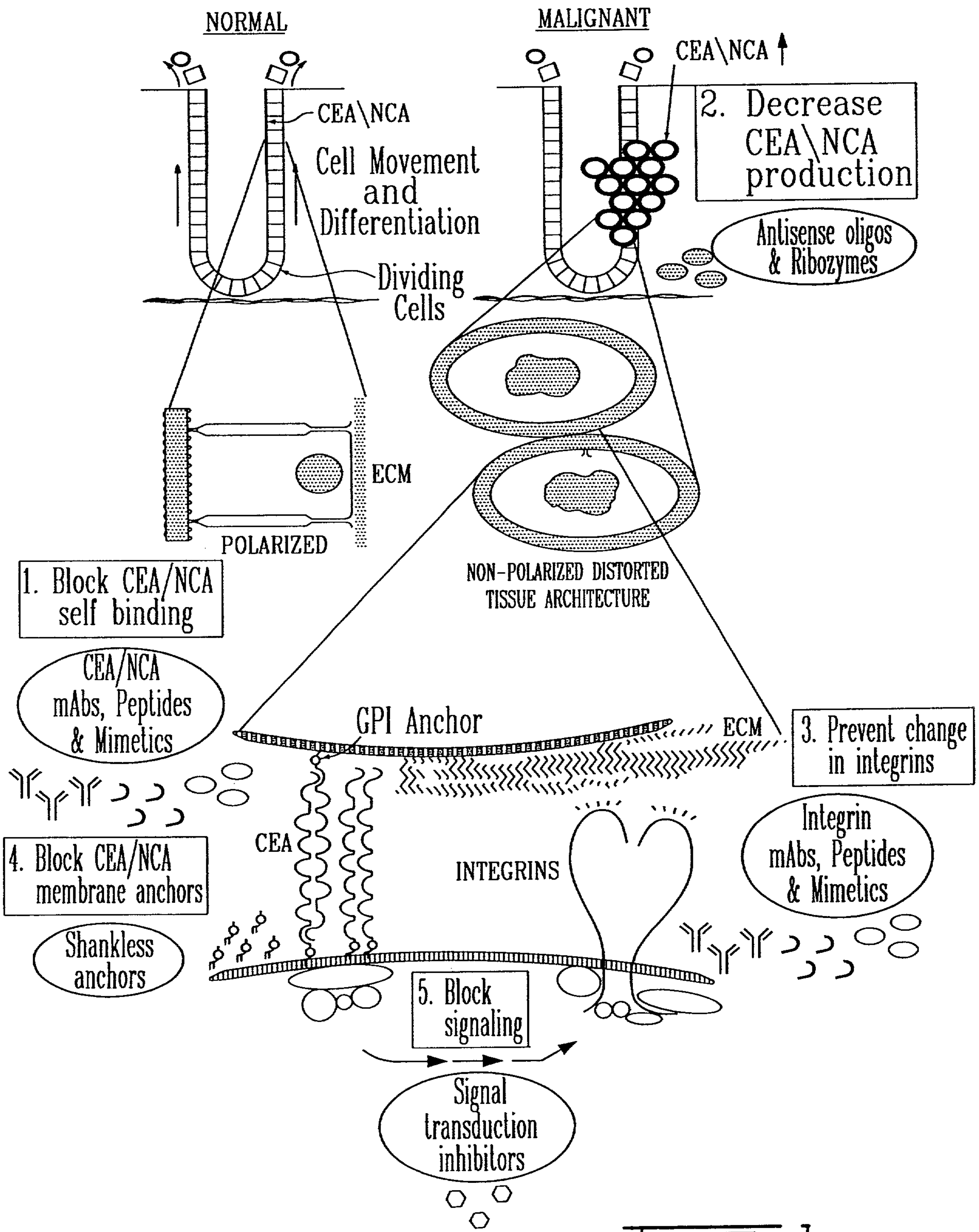


FIG. 1

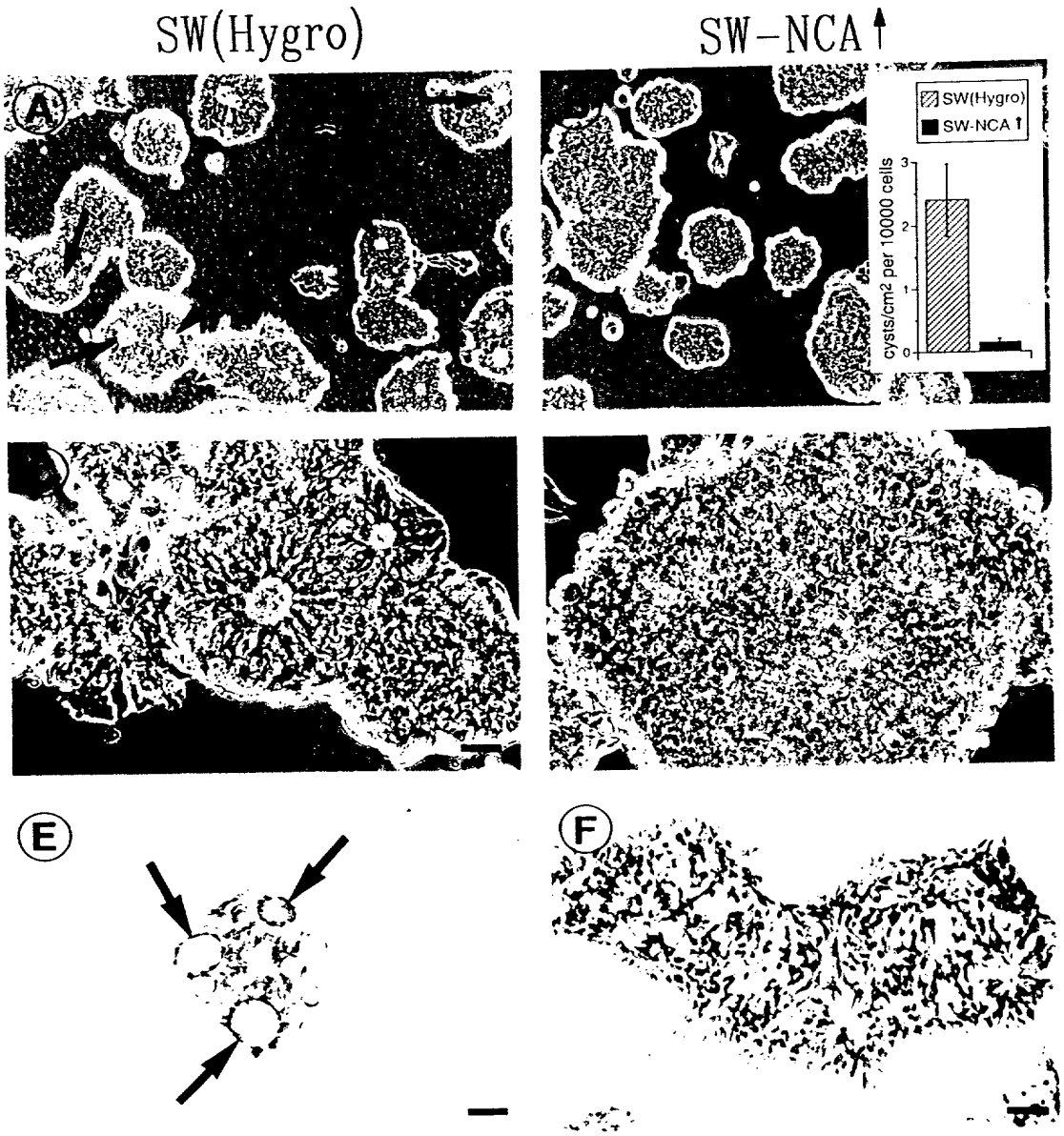


FIG. 2

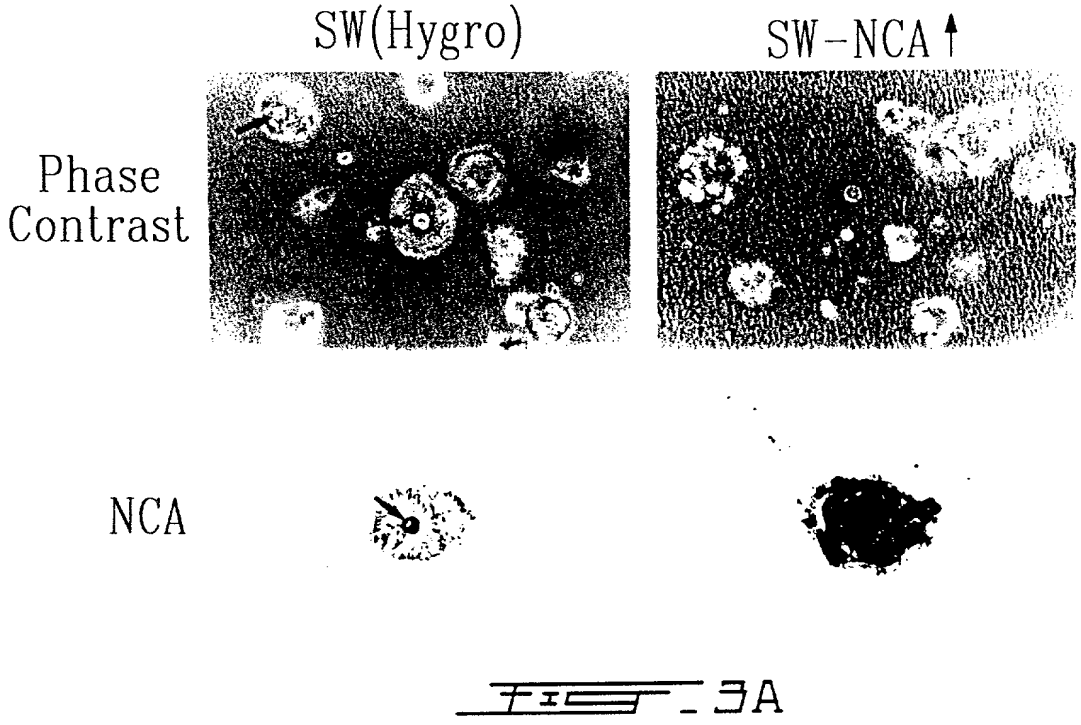


FIG 3A

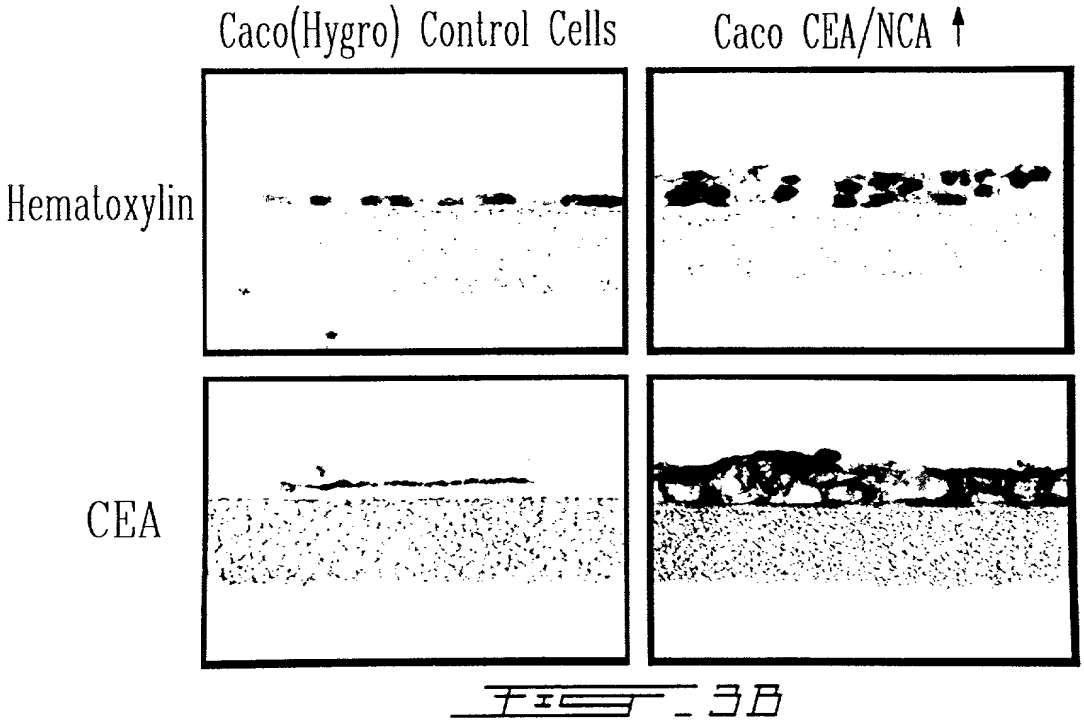
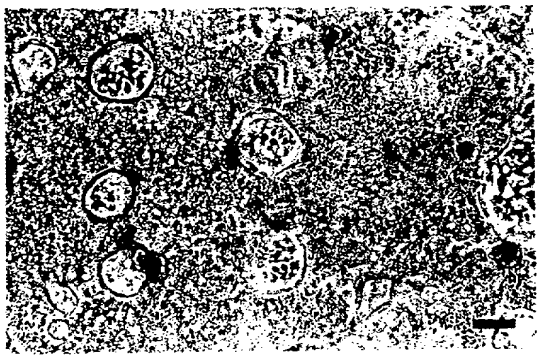


FIG 3B

Caco(Hygro)



Caco CEA/NCA ↑

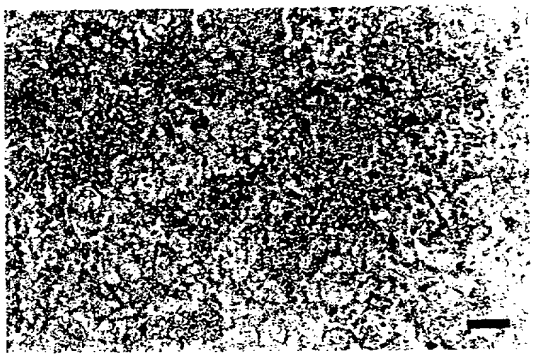


Fig 4A

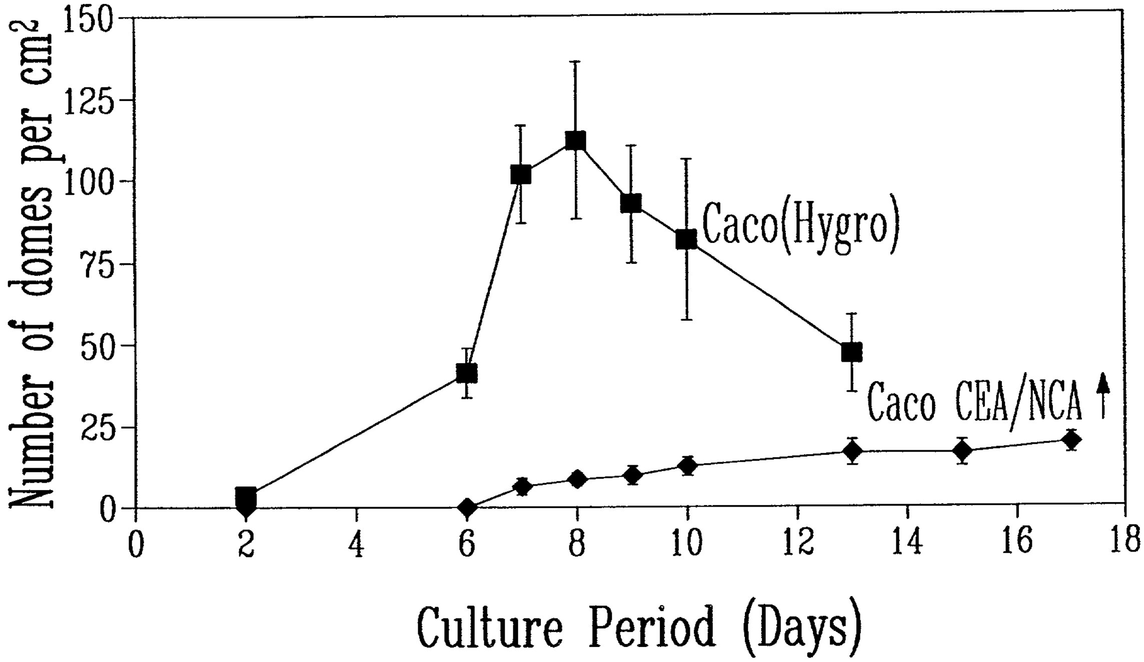

Number of domes per cm²
0
25
50
75
100
125
150
Caco(Hygro)
Caco CEA/NCA ↑
0
2
4
6
8
10
12
14
16
18
Culture Period (Days)

FIG. 4B

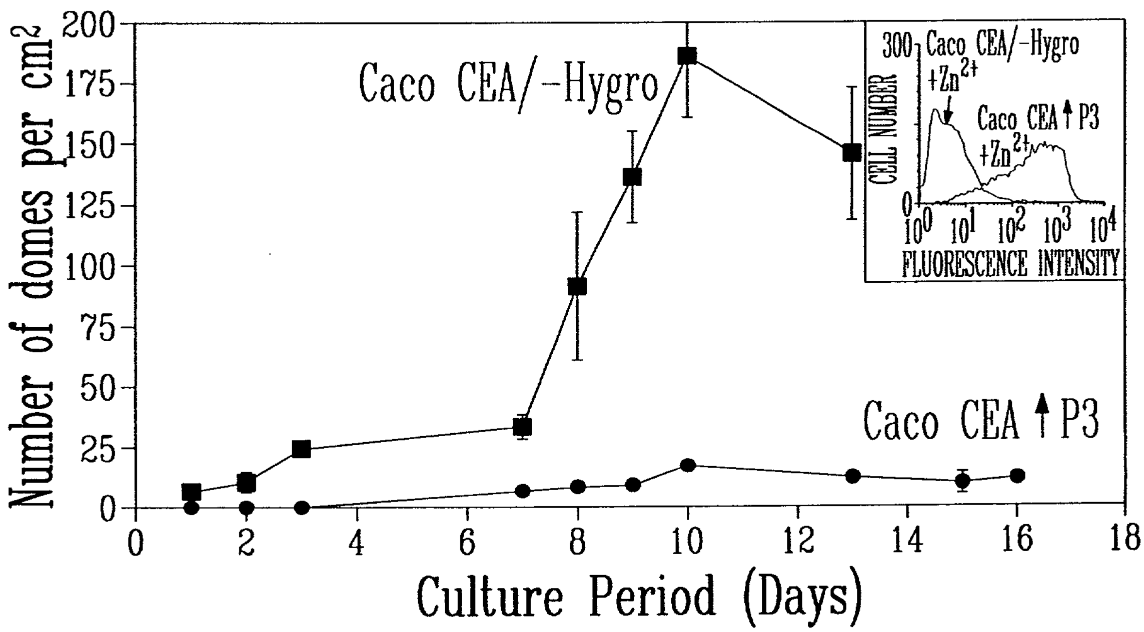

Number of domes per cm²
0
25
50
75
100
125
150
175
200
Caco CEA/-Hygro
Caco CEA ↑ P3
300
0
CELL NUMBER
Caco CEA/-Hygro
+Zn2+
Caco CEA ↑ P3
+Zn2+
10^0 10^1 10^2 10^3 10^4
FLUORESCENCE INTENSITY
0
2
4
6
8
10
12
14
16
18
Culture Period (Days)

FIG. 4C

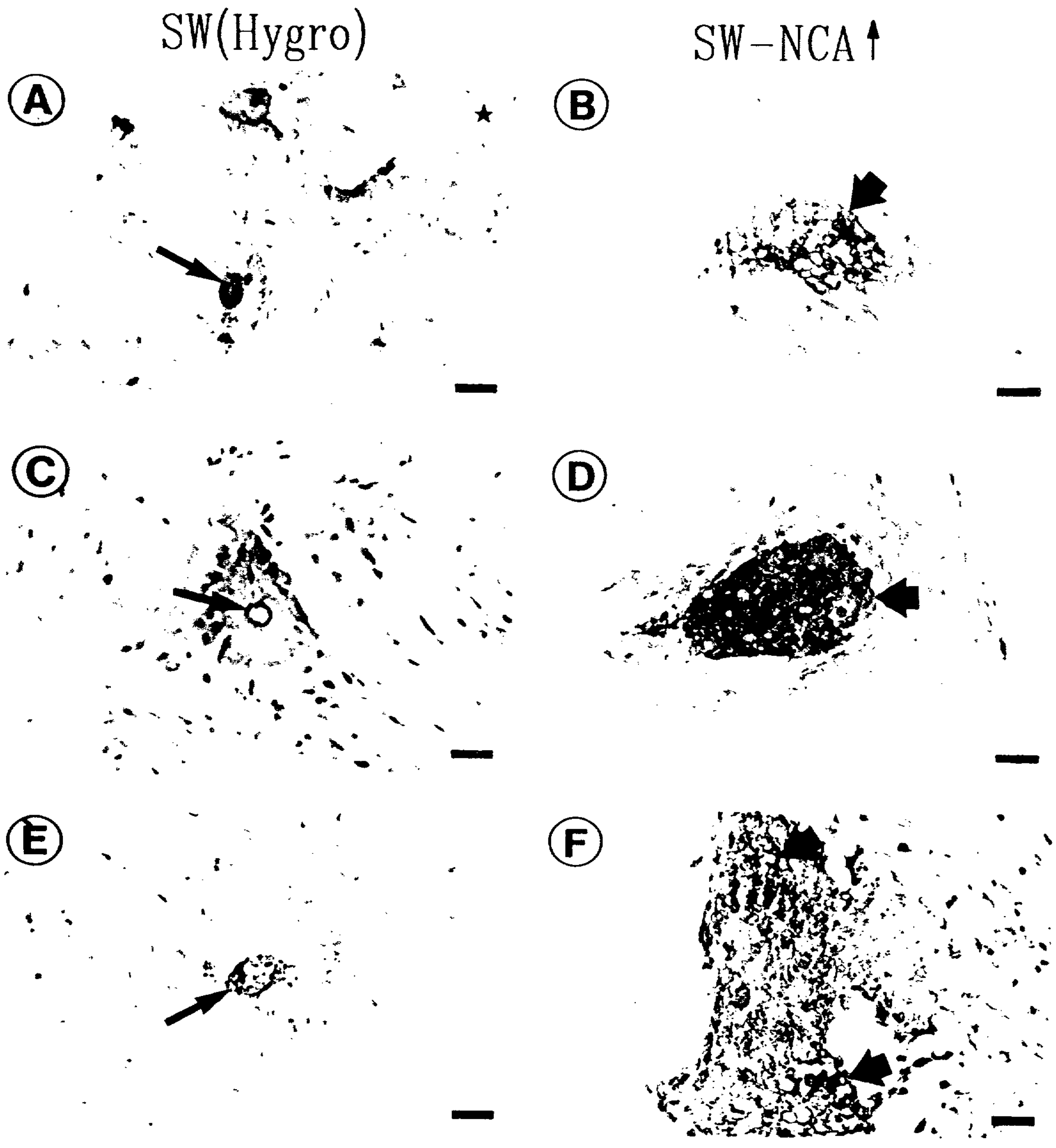


FIG. 5

SW1222
attached

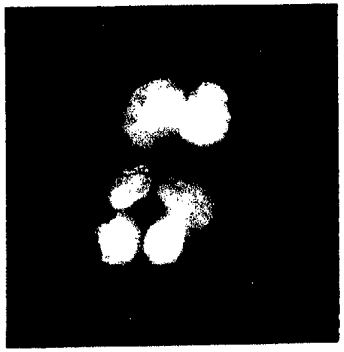


Fig-6A

SW1222
in suspension

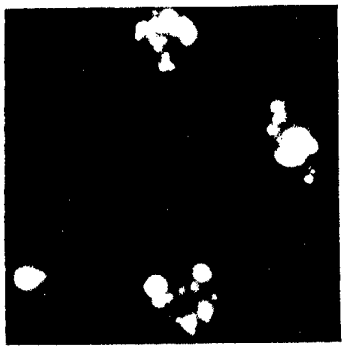


Fig-6B

SW-NCA↑
in suspension

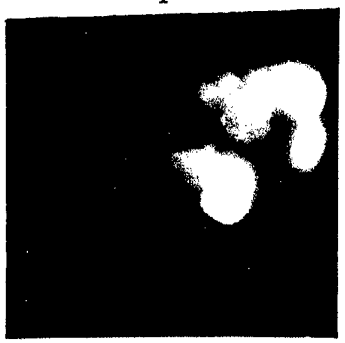


Fig-6C

SW-CEA↓
in suspension

Fig-6D

SW-(hygro)
in suspension

Fig-6E

SW1222

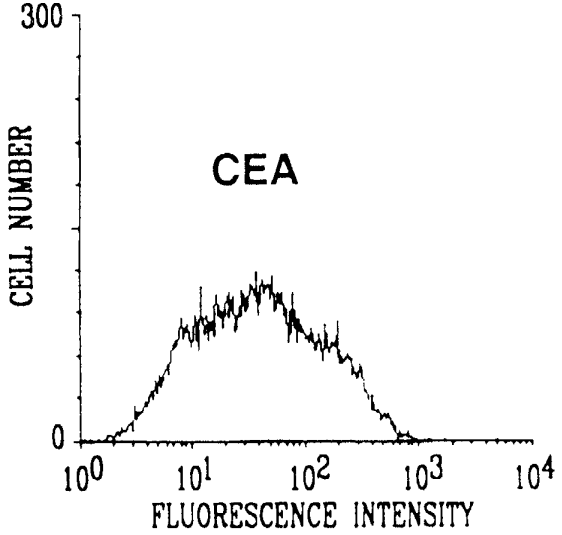
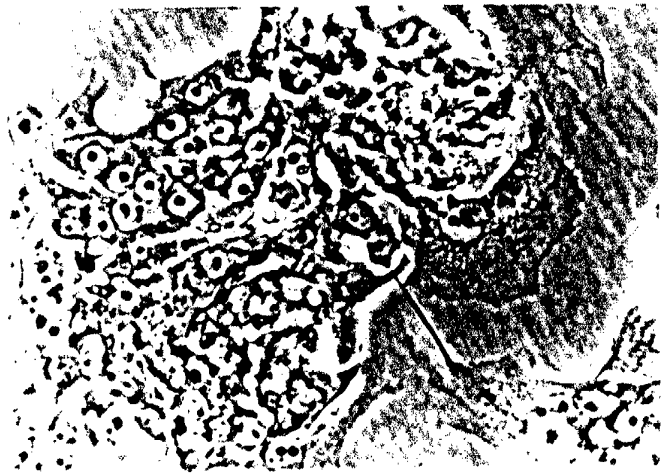


Fig. 7A

SW-CEA ↓



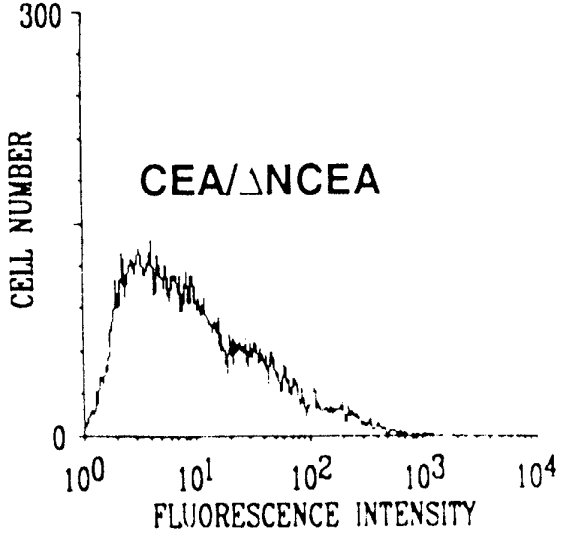


Fig. 7B

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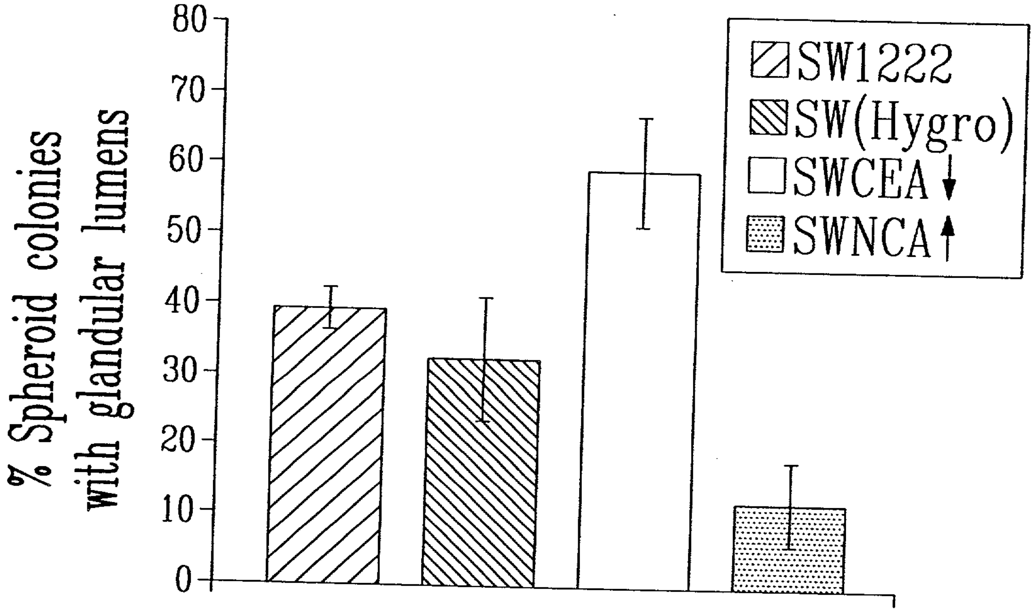
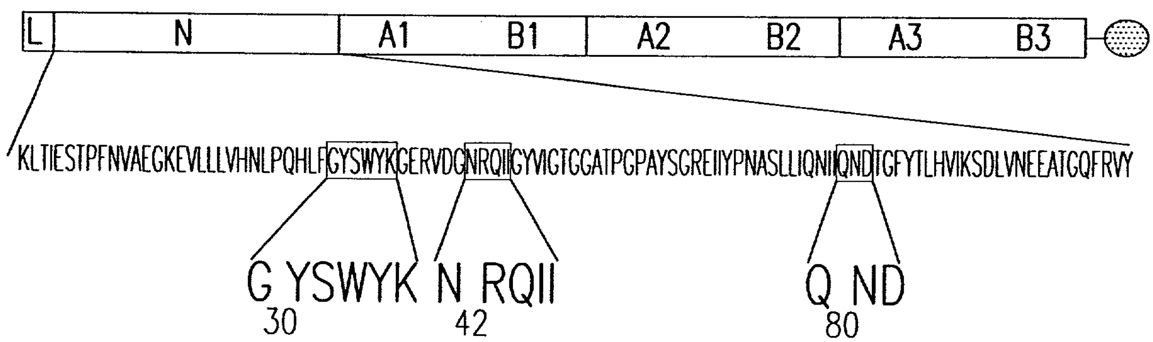


FIG. 8



SUBDOMAINS	MUTATIONS	ADHESION % Single cells (LR)	DIFFERENTIATION % Fusion Index (L6)
–	NONE	17	0
NRQII	NRQII deletion (DNI)	89	51
"	N42D	65	43
"	I45K	12	0
"	I46V	23	0
"	Q44R&I46V	91	100
QND	N80A	56	89
"	N80R	23	81
"	N81A	34	87
"	D82N	27	100
GYSWYK	GYSWYK deletion (DGK)	87	43
"	Y31A	19	2
"	Y34A	74	83
"	Y34F	26	75
"	K35A	86	91

FIG. 9

Control

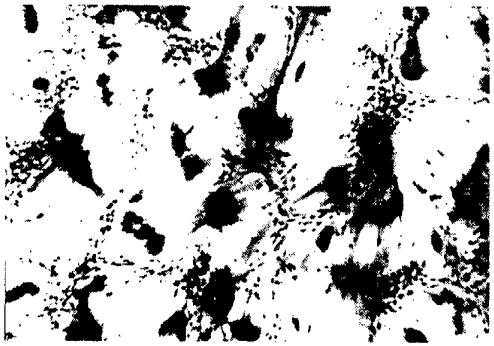


Fig. 10A

CEA

NRQII QND

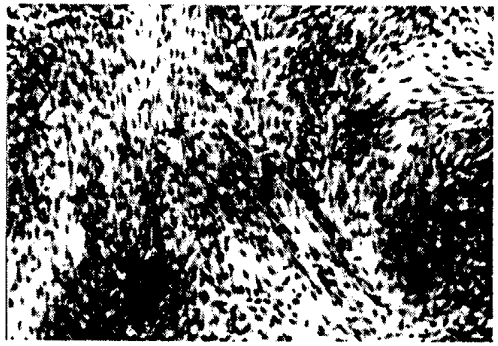


Fig. 10B

ΔNRQII

QND

Fig. 10C

I→V

NRQIV QND

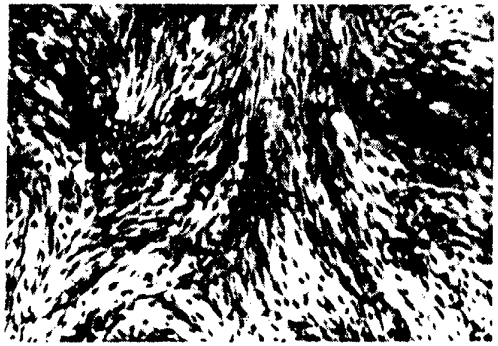


Fig. 10D

Q→R & I→V

NRRIV QND

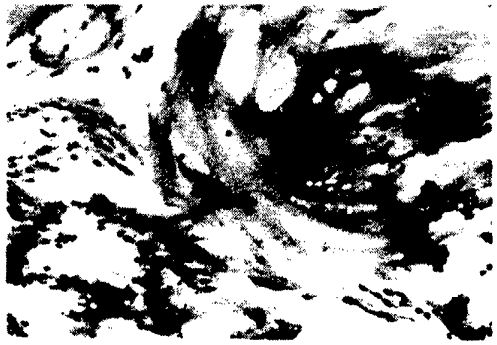


Fig. 10E

D→N

NRQII QNN

Fig. 10F

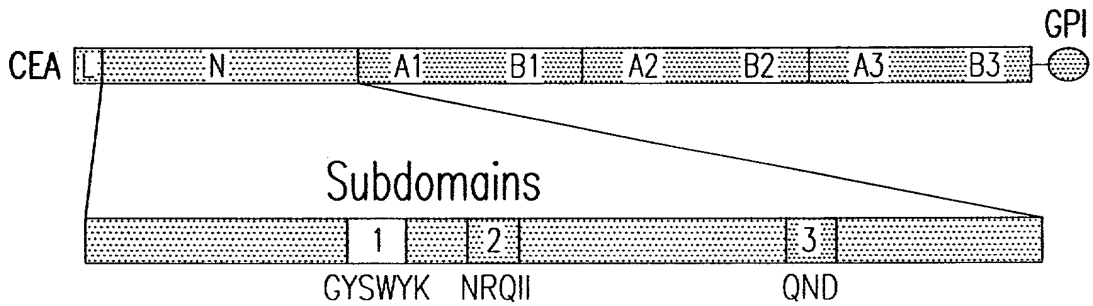


FIG. 11A

Control

Fig - 11B

CEA

Fig - 11C

CEA+ 1

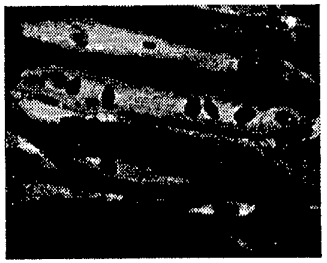


Fig - 11D

CEA+ 2

Fig - 11E

CEA+ 3

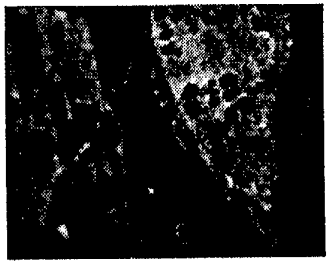


Fig - 11F

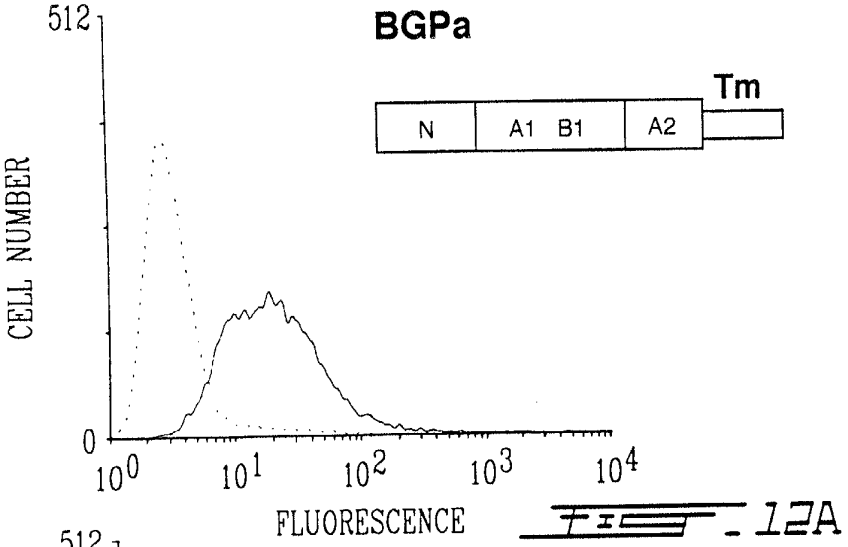


FIG. 12A

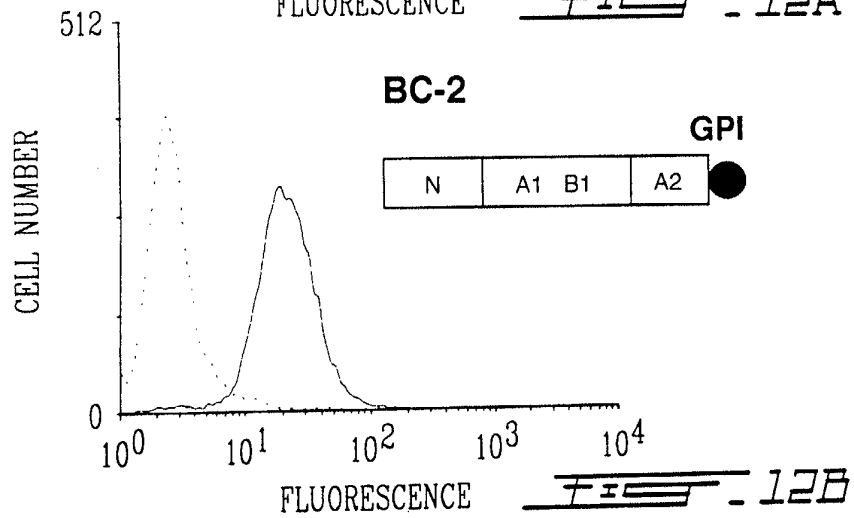
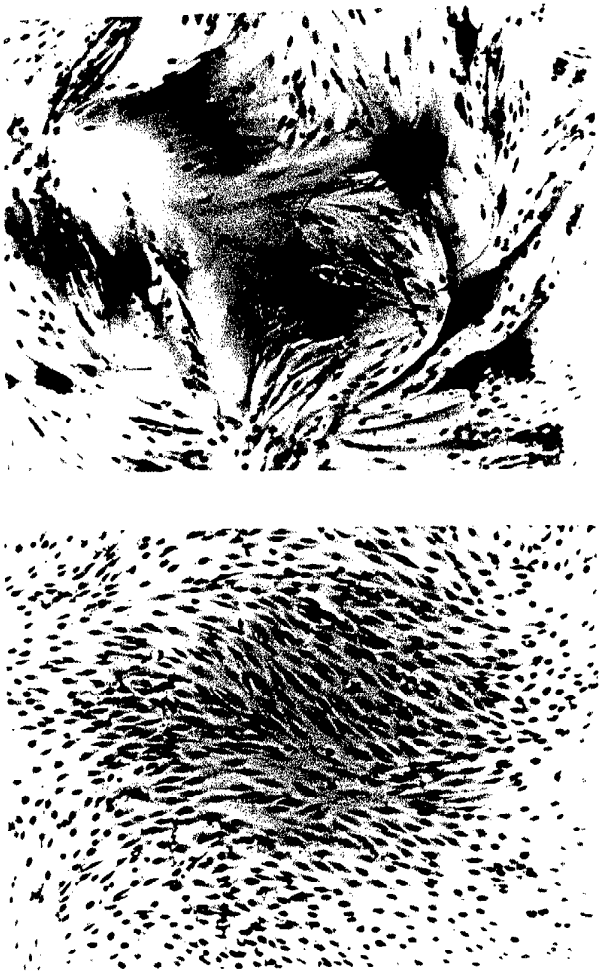


FIG. 12B



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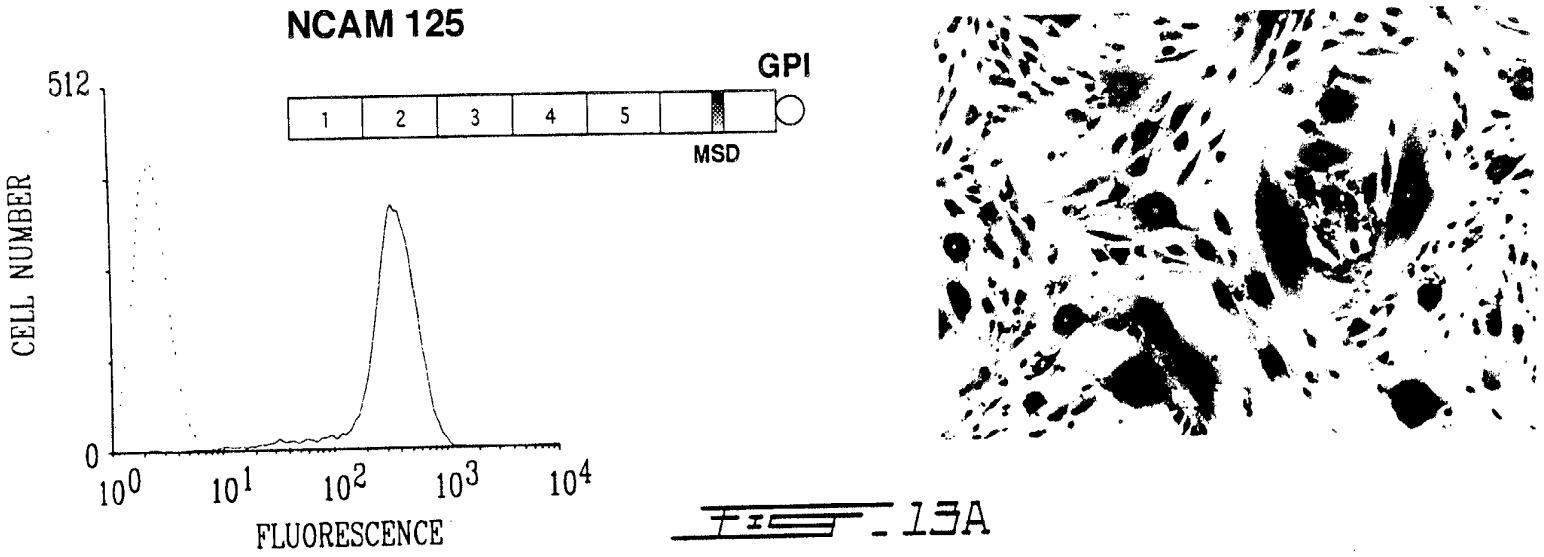

Fig. 13A

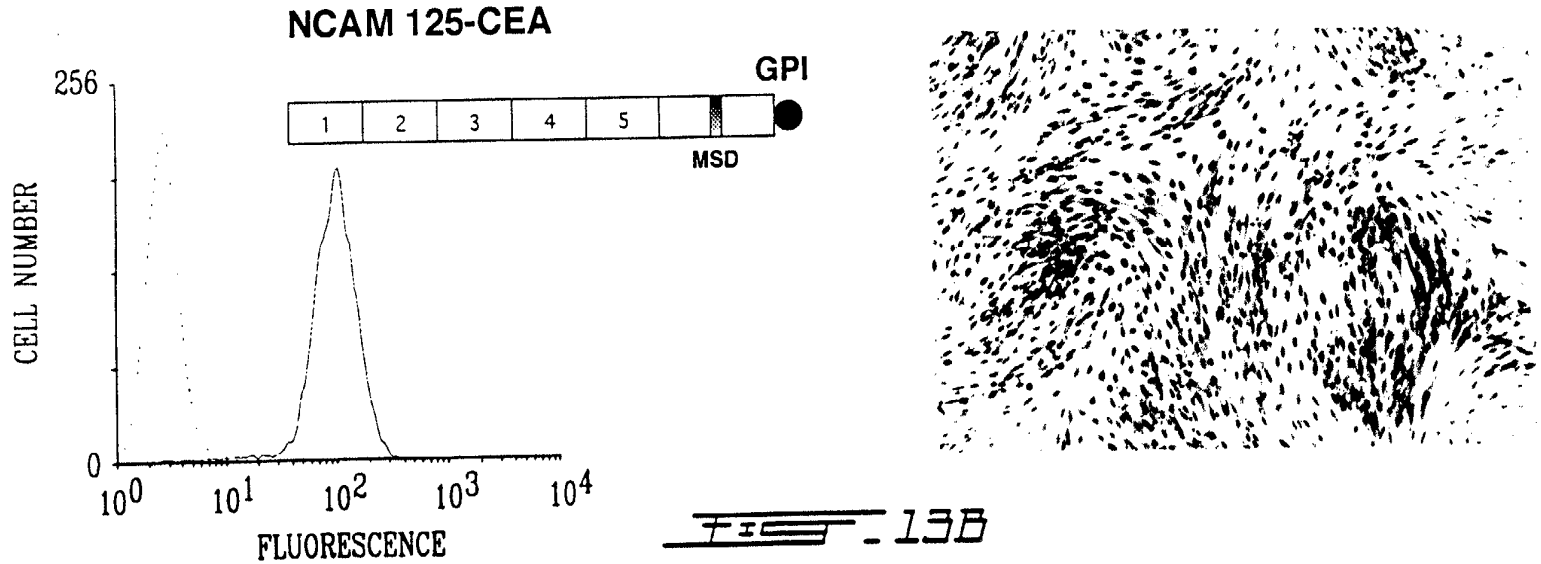

Fig. 13B

SCHWEGMAN ■ LUNDBERG ■ WOESSNER ■ KLUTH

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: **CEA/NCA-BASED DIFFERENTIATION CANCER THERAPY**.

The specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. § 1.56 (attached hereto). I also acknowledge my duty to disclose all information known to be material to patentability which became available between a filing date of a prior application and the national or PCT international filing date in the event this is a Continuation-In-Part application in accordance with 37 C.F.R. § 1.63(e).

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

Foreign application(s), if any, claiming priority under 35 U.S.C. § 119:

Application Number	Country	Day/Month/Year Filed
2,224,129	Canada	12/02/1998

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

No such claim for priority is being made at this time.

I hereby claim the benefit under 35 U.S.C. § 120 or 365(c) of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. § 1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Number	Filing Date	Status
PCT/CA99/00119	February 11, 1999	Pending

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Anglin, J. Michael	Reg. No. 24,916	Jurkovich, Patti J.	Reg. No. 44,813	Nielsen, Walter W.	Reg. No. 25,539
Bianchi, Timothy E.	Reg. No. 39,610	Kalis, Janal M.	Reg. No. 37,650	Oh, Allen J.	Reg. No. 42,047
Billion, Richard E.	Reg. No. 32,836	Kaufmann, John D.	Reg. No. 24,017	Padys, Danny J.	Reg. No. 35,635
Black, David W.	Reg. No. 42,331	Klima-Silberg, Catherine I.	Reg. No. 40,052	Parker, J. Kevin	Reg. No. 33,024
Brennan, Leoniede M.	Reg. No. 35,832	Kluth, Daniel J.	Reg. No. 32,146	Perdok, Monique M.	Reg. No. 42,989
Brennan, Thomas F.	Reg. No. 35,075	Lacy, Rodney L.	Reg. No. 41,136	Prout, William F.	Reg. No. 33,995
Brooks, Edward J., III	Reg. No. 40,925	Lemaire, Charles A.	Reg. No. 36,198	Schumm, Sherry W.	Reg. No. 39,422
Chu, Dinh C.P.	Reg. No. 41,676	LeMoine, Dana B.	Reg. No. 40,062	Schwegman, Micheal L.	Reg. No. 25,816
Clark, Barbara J.	Reg. No. 38,107	Lundberg, Steven W.	Reg. No. 30,568	Scott, John C.	Reg. No. 38,613
Dahl, John M.	Reg. No. 44,639	Maeyaert, Paul L.	Reg. No. 40,076	Smith, Michael G.	Reg. No. 45,368
Drake, Eduardo E.	Reg. No. 40,594	Maki, Peter C.	Reg. No. 42,832	Speier, Gary J.	Reg. No. 45,458
Embretson, Janet E.	Reg. No. 39,665	Malen, Peter L.	Reg. No. 44,894	Steffey, Charles E.	Reg. No. 25,179
Fordenbacher, Paul J.	Reg. No. 42,546	Mates, Robert E.	Reg. No. 35,271	Terry, Kathleen R.	Reg. No. 31,884
Forrest, Bradley A.	Reg. No. 30,837	McCrackin, Ann M.	Reg. No. 42,858	Tong, Viet V.	Reg. No. 45,416
Gamon, Owen J.	Reg. No. 36,143	Moore, Charles L., Jr.	Reg. No. 33,742	Viksnins, Ann S.	Reg. No. 37,748
Harris, Robert J.	Reg. No. 37,346	Nama, Kash	Reg. No. 44,255	Woessner, Warren D.	Reg. No. 30,440
Huebsch, Joseph C.	Reg. No. 42,673	Nelson, Albin J.	Reg. No. 28,650		

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/organization/who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Schwegman, Lundberg, Woessner & Kluth, P.A. to the contrary.

Please direct all correspondence in this case to **Schwegman, Lundberg, Woessner & Kluth, P.A.** at the address indicated below:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Christian Ilantzis

X Additional inventors are being named on separately numbered sheets, attached hereto.

Attorney Docket No.: 186.009US1
Serial No. not assigned
Filing Date: not assigned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Robert A. Screaton

Full Name of inventor:
Citizenship: Residence:
Post Office Address:

Signature: _____ Date: _____

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application:

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.